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(54) Title: ENZYMATIC ANTIMICROBIAL COMPOSITIONS

(57) Abstract

An antimicrobial composition is provided, comprising a Vanadium haloperoxidase, a source of halide and hydrogen peroxide or a source of hydrogen peroxide. Preferably, the Vanadium haloperoxidase is a chloroperoxidase obtainable from *Curvularia inaequalis*.

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ENZYMATIC ANTIMICROBIAL COMPOSITIONS

TECHNICAL FIELD

The present invention relates to the field of enzymatic antimicrobial compositions and their use. More in particular, it relates to enzymatic antimicrobial compositions comprising a Vanadium haloperoxidase, a source of hydrogen peroxide and a source of halide. The invention also relates to the production of Vanadium haloperoxidase by means of recombinant DNA technique, which may be used in enzymatic antimicrobial compositions.

BACKGROUND AND PRIOR ART

Various enzymatic antimicrobial compositions are known in the art. For instance, WO-A-94/04217 discloses stabilized dentifrice compositions which are capable of producing antimicrobially effective concentrations of hypothiocyanite ions ($OSCN^-$). The compositions contain an oxidoreductase to produce hydrogen peroxide and a peroxidase enzyme capable of oxidising thiocyanate ions, which are normally present in saliva, to antimicrobial hypothiocyanite ions ($OSCN^-$). Suitable peroxidases include lactoperoxidase, myeloperoxidase salivary peroxidase and chloroperoxidase.

Enzymatic antimicrobial compositions comprising a haloperoxidase are also disclosed in EP-A-500 387 (Exoxemis). It is described that haloperoxidases selectively bind to and inhibit the growth of target microbes in the presence of peroxide and halide. As suitable haloperoxidases EP-A-500 387 mentions myeloperoxidase (MPO), eosinophil oxidase (EPO), lactoperoxidase (LPO) and chloroperoxidase (CPO). It was reported that the ratio of halide to hydrogen peroxide is a critical factor with regard to the stability and functionality of the haloperoxidase. At very low ratios, hydrogen peroxide can inhibit the haloperoxidase function, whereas at very high ratios, the halide can block the

enzymatic reaction. The ratio may vary over a broad range, but is preferably kept above about 50.

Because of unwanted side reactions of the hydrogen peroxide, the actual hydrogen peroxide concentration in the antimicrobial composition might be lower than expected. Therefore, the present inventors found it more desirable to have the possibility to employ a high starting concentration hydrogen peroxide in the antimicrobial composition, in combination with a conventional amount of halide.

Furthermore, there is a need for enzymatic antimicrobial compositions having a spectrum of antimicrobial activity which is different from that of the known enzymatic antimicrobial compositions. Preferably, the compositions should be capable of exhibiting antimicrobial activity against micro-organisms which are difficult to combat, e.g. Streptococcus faecalis. In other circumstances it may be desirable to combat also non-pathogenic microorganisms, because they may cause spoilage of food products.

Thus, the purpose of the present invention is to provide an enzymatic antimicrobial composition which obviates one or more of the above disadvantages.

We have now surprisingly found that particularly effective enzymatic antimicrobial compositions can be formulated when Vanadium haloperoxidases are used.

The prior art mentioned above includes a variety of haloperoxidases for use in antimicrobial compositions, but no particular attention has been paid so far to the class of Vanadium haloperoxidases.

Vanadium haloperoxidases are different from other haloperoxidases in that the prosthetic group in these enzymes has structural features similar to vanadate (vanadium V), whereas the other haloperoxidases are heme-peroxidases.

A further purpose of the invention was to clone the gene encoding a Vanadium haloperoxidase and to determine its sequence to allow its expression in other

micro-organisms which are more convenient to grow and also to increase the amount of enzyme which can be produced using recombinant DNA techniques.

Some of the Vanadium bromoperoxidases are found in nature on the surface of seaweeds (Wever et al. 1991). In the intact plant in seawater the Vanadium bromoperoxidase is accessible to added substrates and is able to release HOBr upon addition of hydrogen peroxide. The role of the enzyme has not been established, but it is likely that formation of the highly oxidizing HOBr in seawater is part of a defense system of the plant to prevent microbial or fungal growth on its surface.

After the discovery of the non-heme Vanadium bromoperoxidases from the seaweed _Ascophyllum nodosum_, it was shown that a large number of other seaweed species were also contains these enzymes. In particular, the bromoperoxidase from _A. nodosum_ has been extensively studied and characterized (for reviews, see references 2-3). The prosthetic group in these enzymes has structural features similar to vanadate (Vanadium V). In the catalytic mechanism that was derived for this enzyme, hydrogen peroxide reacts with the enzyme to form a hydrogen peroxide-enzyme complex after which bromide and a proton reacted with the complex to form an enzyme-HOBr complex. It was shown (De Boer et al., 1988) that this complex decayed to yield enzyme and HOBr. These vanadium bromo-peroxidases were also shown (De Boer et al., 1988)) to have a high operational stability in aqueous and organic media. For example, these enzymes were stable for three weeks under turnover conditions and could be stored for more than a month in organic solvents such as acetone, methanol, ethanol (present up to 60% v/v) and 1-propanol, without loss of activity. However, these enzymes have the disadvantage that for potential applications bromide should be present or added and further attempts to clone the genes coding for these bromo peroxidases from seaweeds and to determine their amino acid sequences have not been successful.

The known existing heme-containing chloroperoxidase from Caldariomyces fumago is less suitable for preparing enzymatic antimicrobial compositions because of its inherent instability and its low pH optimum of 2.75 (J.R. Kanofsky, 1984), which seriously limits its application. Similar arguments prevent the application of the enzyme myeloperoxidase (MPO) from human white blood cells, which is also capable of generating HOCl (A.R.J. Bakkenist et al., 1980).

Reports (see references 8, 9) have already appeared that dematiaceous hyphomyctes secrete haloperoxidases with pronounced stability. In particular it was shown that the terrestrial fungus Curvularia inaequalis secretes a Vanadium chloroperoxidase (J.W.P.M. Van Schijndel et al, 1993) which possesses a high affinity for chloride and has a pH optimum for the chlorination reaction around pH 5.5. As with the bromoperoxidase from seaweed, the prosthetic group in the chloroperoxidase has structural features similar to vanadate (vanadium V). In a subsequent more detailed study (J.W.P.M. Van Schijndel et al., 1994), it was shown that the enzyme kinetics of the oxidation of chloride by hydrogen peroxide resemble that of the vanadium bromoperoxidase from the seaweed A. nodosum. Further, three different methods showed that the enzyme produces an oxidized chlorine species (HOCl) as reaction product and was by itself resistant towards this product. The enzyme exhibits a high thermostability (Tm 90°C) and displays high stability in organic solvent such as 40 % methanol, ethanol and propanol.

DEFINITION OF THE INVENTION

In a first aspect, the present invention relates to an enzymatic antimicrobial composition comprising a Vanadium haloperoxidase, a source of halide and hydrogen peroxide or a source of hydrogen peroxide.

According to a second aspect, the present invention relates to the production of a Vanadium

haloperoxidase by transforming a suitable host by means of an expression vector comprising an origin of replication, transcription and termination control sequences and at least part of the DNA sequence coding for a Vanadium haloperoxidase, cultivating the host under conditions which allow the expression of the structural gene and isolating the Vanadium haloperoxidase.

DETAILED DESCRIPTION OF THE INVENTION

(a) The Vanadium haloperoxidase

The enzymatic antimicrobial compositions according to the invention comprise, as a first constituent, a Vanadium haloperoxidase. The Vanadium haloperoxidase may in principle be chosen from the various Vanadium haloperoxidases which have been disclosed in the art. For example, one can use the Vanadium (non-heme) haloperoxidase produced from Curvularia inaequalis, such as described in US-A-4 707 466. Alternatively, one may isolate and purify the chloroperoxidase from Curvularia inaequalis (CBS 102.42) according to the method of J.W.P.M. Van Schijndel et al. (1993).

Other sources of Vanadium haloperoxidases include Drechslera biseptata (CBS 371.72), Drechslera fugax (CBS 509.77), Drechslera nicotiae (CBS 655.74), Drechslera subpapendorfii (656.74), Embelisia hyacinthi (416.71), Embelisia didymospora (CBS 766, Ulocladium chartarum (200.67) and Ulocladium botrytis (452.72).

Alternatively, Vanadium haloperoxidase can be prepared by recombinant DNA techniques by transforming a suitable host by means of an expression vector comprising an origin of replication, transcription and termination control sequences and the DNA sequence coding for a Vanadium haloperoxidase, cultivating the host under conditions which allow the expression of the structural gene and isolating the Vanadium haloperoxidase. This is described in detail in the Examples below.

(b) The source of halide ions.

The second constituent of the hygienic compositions according to the invention is a source of halide ions. These may be any halide ions, but a source of iodide or chloride ions are preferred because they are most effective. Sodium chloride is the most preferred source of halide ions. The halide may be added to the enzymatic antimicrobial compositions of the invention, or alternatively, the halide may be used which is naturally present in tap water and which is usually in the order of 2-5 mM.

(c) The source of hydrogen peroxide.

The hygienic compositions according to the invention further comprise a source of hydrogen peroxide, or a hydrogen peroxide-generating system. Examples of suitable hydrogen peroxide-generating systems are perborate or percarbonate salts, preferably sodium percarbonate or sodium perborate.

The hydrogen peroxide may also be provided by an enzymatic hydrogen peroxide generating system. The enzymatic hydrogen peroxide-generating system may in principle be chosen from the various enzymatic hydrogen peroxide-generating systems which have been disclosed in the art. For example, one may use an amine oxidase and an amine, an amino acid oxidase and an amino acid, lactate oxidase and lactate, cholesterol oxidase and cholesterol, uric acid oxidase and uric acid or a xanthine oxidase with xanthine. Preferred, however, is the combination of glucose oxidase and glucose.

The amount of glucose oxidase will depend on its specific activity and the activity of any residual catalase that may be present, but by way of example it can be stated generally that the detergent composition according to the invention will contain from 10 to 1000, preferably from 20 to 500 units glucose oxidase per g or ml of the detergent composition, a unit of enzyme activity being defined as the

quantity required to convert 1 μmol of substrate per minute under standard conditions.

(d) Other ingredients.

The enzymatic antimicrobial compositions of the invention generally comprise from 0.01 to 50 % by weight, preferably 0.1 to 5.0% by weight of one or more surfactants are wetting agents. Suitable surfactants or detergent-active compounds are soap or non-soap anionics, nonionics, cationics, amphoteric or zwitterionic compounds. The surfactant system usually comprises one or more anionic surfactants and one or more nonionic surfactants. The surfactant system may additionally contain amphoteric or zwitterionic detergent compounds, but this is not normally desired owing to their relatively high cost.

In general, the nonionic and anionic surfactants of the surfactant system may be chosen from the surfactants described "Surface Active Agents" Vol. 1, by Schwartz & Perry, Interscience 1949, Vol. 2 by Schwartz, Perry & Berch, Interscience 1958, in the current edition of "McCutcheon's Emulsifiers and Detergents" published by Manufacturing Confectioners Company or in "Tenside-Taschenbuch", H. Stache, 2nd Edn., Carl Hauser Verlag, 1981.

Suitable nonionic detergent compounds which may be used include, in particular, the reaction products of compounds having a hydrophobic group and a reactive hydrogen atom, for example, aliphatic alcohols, acids, amides or alkyl phenols with alkylene oxides, especially ethylene oxide either alone or with propylene oxide. Specific nonionic detergent compounds are C_6-C_{22} alkyl phenol-ethylene oxide condensates, generally 5 to 25 EO, i.e. 5 to 25 units of ethylene oxide per molecule, and the condensation products of aliphatic C_8-C_{18} primary or secondary linear or branched alcohols with ethylene oxide, generally 5 to 40 EO.

Suitable anionic detergent compounds which may be used are usually water-soluble alkali metal salts of organic sulphates and sulphonates having alkyl radicals containing from about 8 to about 22 carbon atoms, the term alkyl being used to include the alkyl portion of higher acyl radicals. Examples of suitable synthetic anionic detergent compounds are sodium and potassium alkyl sulphates, especially those obtained by sulphating higher C_8-C_{18} alcohols, produced for example from tallow or coconut oil, sodium and potassium alkyl C_9-C_{20} benzene sulphonates, particularly sodium linear secondary alkyl C_{10}-C_{15} benzene sulphonates; and sodium alkyl glyceryl ether sulphates, especially those ethers of the higher alcohols derived from tallow or coconut oil and synthetic alcohols derived from petroleum. The preferred anionic detergent compounds are sodium C_{11}-C_{15} alkyl benzene sulphonates and sodium C_{12}-C_{18} alkyl sulphates.

Also applicable are surfactants such as those described in EP-A-328 177 (Unilever), which show resistance to salting-out, the alkyl polyglycoside surfactants described in EP-A-070 074, and alkyl monoglycosides.

Preferred surfactant systems are mixtures of anionic with nonionic detergent active materials, in particular the groups and examples of anionic and nonionic surfactants pointed out in EP-A-346 995 (Unilever). Especially preferred is surfactant system which is a mixture of an alkali metal salt of a C_{16}-C_{18} primary alcohol sulphate together with a C_{12}-C_{15} primary alcohol 3-7 EO ethoxylate.

The nonionic detergent is preferably present in amounts greater than 10%, e.g. 25-90% by weight of the surfactant system. Anionic surfactants can be present for example in amounts in the range from about 5% to about 40% by weight of the surfactant system.

The enzymatic detergent compositions of present invention may also comprise other constituents normally used in antimicrobial compositions, such as thickening agents. Particularly useful in this respect are the

combinations of surfactants disclosed in EP-A-314 232 (Unilever), which provide thickening gels upon dissolution with water.

The antimicrobial compositions of the invention may be employed to provide hygiene benefits for hard-surface cleaning and fabric washing, but also to provide hygiene and cleaning in industrial/institutional applications such as in hospitals and for cleaning and disinfecting medical equipment. Another application is in the dairy industry, for disinfecting milking equipment. The antimicrobial compositions can also be succesfully used in deodorants in view of their ability to combat bacteria which cause malodour.

The antimicrobial compositions of the invention may be used in the form of powders which are to be dissolved in water before use, but the can also be formulated as liquids products or gels. In those product forms it is important that the production of hypohalite is not initiated until the composition is used. This can be achieved by physically separating the enzyme and its substrate, e.g. by encapsulating the enzyme according to well known techniques.

When the enzymatic antimicrobial composition is used, it is diluted 5 to 100 times by addition of water to provide a medium having an effective antimicrobial activity. It is then brought into contact with the surface to be disinfected and allowed

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The invention will be further illustrated by means of the following non-limiting Examples.

EXAMPLE 1

Minimal Inhibitory Concentration (MIC) of hypochlorite.

Materials:

Bacterial strains used in this example were Escherichia coli NCTC 900, Pseudomonas aeruginosa ATCC 15442, Staphylococcus aureus ATCC 13565, Streptococcus faecalis NCTC 1092 and Listeria innocua ATCC 33090 serotype 6B. Bacteria were grown for 15 to 18 hours at 30°C in Brain Heart Infusion (BHI) broth-medium. After cultivation the strains were washed two times in citrate buffer pH 5.5 (20 mM Na_3citrate-NaOH buffer + 10 mM NaCl). The bacterium suspensions were centrifuged in an Eppendorf centrifuge (14,000 rpm for 5 min), then the supernatants were removed and subsequently the bacterial pellet was resuspended in citrate buffer. This washing procedure was then repeated once more for each bacterial suspension. The twice washed bacterial suspension was then diluted with citrate buffer pH 5.5 to obtain a suspension of approximately 10^7 bacteria per ml. During the washing steps the cells were kept on ice. Buffers and BSA-solution (1% w/v in citrate buffer pH 5.5) were filter-sterilised and stored at 4°C. The hypochlorite solutions were made from a stock-solution (107,000 ppm) by dilution with sterile demineralised water.

Methodology:

Using sterile techniques, a suspension of approximately 10^7 bacteria per ml in citrate buffer pH 5.5 was made. From this suspension aliquots of 1.9 ml were added to sterile tubes. Subsequently 0.1 ml of cold hypochlorite solutions of various concentrations were added to the tubes, in such a way that a hypochlorite dilution range is obtained. The tubes were mixed continuously with a magnetic stirrer. Also

blank determinations were included where only sterile buffer was added instead of a hypochlorite solution. The samples were incubated for exactly 5 min at 30°C. After this incubation period 1 ml of the reaction mixture was taken and added to 9 ml cold BSA (1% w/v) solution and put on ice immediately. This was done to stop the reaction of hypochlorite with the microorganisms. Survival as Colony Forming Units (CFU) per ml was determined by diluting the sample from 10^{-1} to 10^{-5} and plating 100 μl samples of the various dilutions on labelled BHI-agar plates. The plates were then incubated for 15-18 hours at 30°C. When there were no CFU detectable after this incubation period, the plates are incubated for another 24 hours at 30°C.

Definition: the Minimal Inhibitory Concentration (MIC-value) is defined here as the concentration of hypochlorite which leads under the experimental conditions used to at least a log 6 reduction in colony forming units of the specific microorganism tested.

The results obtained are shown in Table I. The values found are in the same range as reported in the literature.

Table I. MIC values for various microorganisms.

Microorganism	MIC-value in ppm
<u>Escherichia coli</u>	2-3
<u>Staphylococcus aureus</u>	2-3
<u>Listeria innocua</u>	2-3
<u>Pseudomonas aeruginosa</u>	2-3
<u>Streptococcus faecalis</u>	2-3

EXAMPLE 2

Minimal Inhibitory Concentration of hypochlorite and hypochlorite generated enzymatically by vanadium-chloroperoxidase (V-CPO) from *Curvularia inaequalis*.

In this example the killing effect of hypochlorite is compared to the killing effect of hypochlorite generated enzymatically by vanadium-chloroperoxidase (V-CPO) from *Curvularia inaequalis*. In order to make comparisons possible, a micropump was used. This was done to make the hypochlorite concentration during the experiment follow the same profile as in the situation were hypochlorite is generated enzymatically. Also the V-CPO activity was determined very carefully under the conditions of the experiment, this to know the amount of hypochlorite present at each time point of the experiment.

Materials:

Bacterial strains used in this example were *Escherichia coli* NCTC 900, *Pseudomonas aeruginosa* ATCC 15442, *Staphylococcus aureus* ATCC 13565, *Streptococcus faecalis* NCTC 1092 and *Listeria innocua* ATCC 33090 serotype 6B. Bacteria were grown for 15 to 18 hours at 30°C in Brain Heart Infusion (BHI) broth-medium. After cultivation the strains were washed two times in citrate buffer pH 5.5 (20 mM Na_3citrate-NaOH buffer + 10 mM NaCl). The bacterium suspensions were centrifuged in an Eppendorf centrifuge (14000 rpm for 5 min), then the supernatant were removed and subsequently the bacterial pellet was resuspended in citrate buffer. This washing procedure was then repeated once more for each bacterial suspension. The twice washed bacterial suspension was then diluted with citrate buffer pH 5.5 to obtain a suspension of approximately 10^7 bacteria per ml. During the washing steps the cells were kept on ice. Buffers and BSA-solution (1% w/v in citrate buffer pH 5.5) were filter-sterilised and stored at 4°C. The hypochlorite solutions were made from a stock-solution (107,000 ppm) by dilution with sterile demineralised water.

H_2O_2 solutions were made from a 30% stock solution by dilution with sterile demineralised water. Casitone was obtained from Difco. Chloroperoxidase from <u>Curvularia inaequalis</u> was purified according to van Schijdel et al. (1993). Chloroperoxidase activity in the conversion of Cl- to HOCl was determined spectrophotometrically in 20 mM sodium citrate buffer pH 5.5, 10 mM NaCl, 100 μM H_2O_2, 50 μM monochlorodimedone at 30°C, by following the conversion of monochlorodimedone (e 290 nm = 20.2 $mM^{-1}.cm^{-1}$) to dichlorodimedone (e 290 nm = 0.2 $mM^{-1}.cm^{-1}$). 1 Unit of chloroperoxidase is defined as the amount of enzyme that converts 1 μmol of monochlorodimedone (Sigma) per minute.

Methodology:
Using sterile techniques, a suspension of approximately 10^7 bacteria per ml in citrate buffer pH 5.5 was made. From this suspension aliquots of 1.8 ml were added to a sterile reaction vessel, which was continuously stirred throughout the experiment with a magnetic stirrer. Subsequently 0.2 ml of V-CPO solutions of various concentrations (for calculations, see below) were added to the reaction vessels, in such a way that a V-CPO dilution range is obtained. Also blank determinations were included where 0.2 ml sterile buffer was added instead 0.2 ml of V-CPO solution. The reaction vessels was incubated at 30°C. Then 0.5 ml of a H_2O_2 stock solution was added, to start the V-CPO reaction. The H_2O_2 stockconcentration used depended on the hypochlorite concentration produced, and was chosen in such a way that the endconcentration of H_2O_2 (at the end of the flux) was a fivefold molar excess compared to the endconcentration of hypochlorite. The samples were incubated for exactly 5 min at 30°C. After this incubation period 1 ml of the reaction mixture was taken and added to 9 ml cold BSA (1% w/v) solution and put on ice immediately. This was done to stop the reaction of hypochlorite with the microorganisms. Survival as Colony Forming Units (CFU) per ml was determined by diluting the sample from 10^{-1} to 10^{-5} and plating 100 μl samples of the various dilutions on

labelled BHI-agar plates. The plates were then incubated for 15-18 hours at 30°C. When there were no CFU detectable after this incubation period, the plates are incubated for another 24 hours at 30°C.

The MIC value obtained was compared to the MIC value that was obtained when the same hypochlorite amounts were added, using a micropump. This was done as follows:

Using sterile techniques, a suspension of approximately 10^7 bacteria per ml in citrate buffer pH 5.5 was made. From this suspension aliquots of 1.8 ml were added to a sterile reaction vessel, which was continuously stirred throughout the experiment with a magnetic stirrer. The reaction vessels were incubated at 30°C. Then 0.2 ml of a H_2O_2 stock solution was added. The H_2O_2 stock concentration used depended on the hypochlorite concentration produced, and was chosen in such a way that the end concentration of H_2O_2 (at the end of the flux) was a five-fold molar excess compared to the endconcentration of hypochlorite. Then a flux of 0.5 ml of a hypochlorite solutions of a known concentration was applied over 5 min (flow: 0.1 ml per min) at 30°C. A series of experiments were done, each using various hypochlorite solutions of a different concentration, so that a range of hypochlorite end concentrations was obtained (for calculations see below). After the incubation period of 5 minutes, 1 ml of the reaction mixture was taken and added to 9 ml cold BSA (1% w/v) solution and put on ice immediately. This was done to stop the reaction of hypochlorite with the microorganisms. Survival as Colony Forming Units (CFU) per ml was determined by diluting the sample from 10^{-1} to 10^{-5} and plating 100 μl samples of the various dilutions on labelled BHI-agar plates. The plates were then incubated for 15-18 hours at 30°C. When there were no CFU detectable after this incubation period, the plates are incubated for another 24 hours at 30°C.

The similarity of the hypochlorite profiles obtained in the experiments described above with V-CPO respectively with hypochlorite from a stock solution were confirmed by using the same experimental set-up (as for the blank experiments without the microorganisms added) coupled to a spectrophotometer, in which the hypochlorite concentrations in time could be followed at 290 nm, making use of the conversion of monochlorodimedone (e 290nm = 20.2 $mM^{-1}.cm^{-1}$) to dichlorodimedone (e 290nm = 0.2 $mM^{-1}.cm^{-1}$).

Calculations:
The endconcentration of hypochlorite generated with V-CPO was calculated as follows:
With 0.01 U per ml of V-CPO 0.01 µmol of hypochlorite per ml per min is generated, which is equivalent to 0.05 µmol hypochlorite per ml per 5 min. due to the dilution effect of the flux of 0.5 ml, which is added to 2.0 ml initial volume, the endconcentration after 5 minutes is (2.0/2.5) * 0.05 µmol per ml = 0.04 µmol hypochlorite per ml after 5 minutes. This concentration could subsequently be expressed as 0.04 µmol hypochlorite per ml = 0.04 * 52.5 ppm hypochlorite = 2.10 ppm hypochlorite. Other hypochlorite endconcentrations were obtained by increasing or decreasing the V-CPO concentration. The endconcentration of hypochlorite added was calculated as follows:
Since a flux of 0.5 ml is added to a reaction volume of 2.0 ml, the endvolume is 2.5 ml, which corresponds to diluting fivefold. In order to obtain a hypochlorite end concentration of 2.10 ppm, a stock solution of 10.50 ppm was used. Other hypochlorite concentrations were obtained by increasing or decreasing the hypochlorite stock solution.

Definition: the Minimal Inhibitory Concentration (MIC-value) is defined here as the concentration of hypochlorite which leads under the experimental conditions used to at least a log 6 reduction in colony forming units of the specific microorganism tested.

The results are shown in Table II. The killing effects of various endconcentrations of hypochlorite, either produced by V-CPO or at the end of- the hypochlorite flux, are compared. As can be concluded from Table II, hypochlorite produced enzymatically by V-CPO already gives total inhibition of growth at hypochlorite concentrations as low as 0.4 ppm for all organisms tested except for Staphylococcus aureus, whereas 2 ppm hypochlorite is needed to obtain the same growth inhibition. This shows that vanadium-containing haloperoxidase is a more efficient hygienic component than would have been expected from just its hypochlorite producing capacity. Also it is apparent that V-CPO gives killing of all microorganisms tested, pathogenic or non-pathogenic, whereas heme-containing haloperoxidase are claimed to give only efficient killing of pathogenic bacteria (see EP-A-500 387).

Table II. MIC values for various microorganisms.

Microorganism	MIC-value with hypochlorite (in ppm)	MIC-value with V-CPO (in ppm)
Escherichia coli	2	0.4
Staphylococcus aureus	2-3	1.6
Listeria innocua	2	0.4
Pseudomonas aeruginosa	2	0.4
Streptococcus faecalis	2	0.4

EXAMPLE 3

Killing efficiencies of hypochlorite and hypochlorite generated enzymatically by V-CPO in the presence of protein hydrolysate.

For obtaining hygiene it is known that in situations encountered in practice, overdosing of hypochlorite is necessary, since this reactive molecule will not only react with microorganisms, but also with other compounds that are present. Therefor it was important to test the behaviour of vanadium-containing haloperoxidase in a fluid containing e.g. a protein hydrolysate.

Materials:
The bacterial strain used in this example was Escherichia coli NCTC 900. Bacteria were grown for 15 to 18 hours at 30°C in Brain Heart Infusion (BHI) broth-medium. After cultivation the strains were washed two times in citrate buffer pH 5.5 (20 mM Na_3citrate-NaOH buffer + 10 mM NaCl). The bacterium suspensions were centrifuged in an Eppendorf centrifuge (14,000 rpm for 5 min), then the supernatants were removed and subsequently the bacterial pellet was resuspended in citrate buffer. This washing procedure was then repeated once more for each bacterial suspension. The twice washed bacterial suspension was then diluted with citrate buffer pH 5.5 to obtain a suspension of approximately 10^8 bacteria per ml. During the washing steps the cells were kept on ice. Buffers and BSA-solution (1% w/v in citrate buffer pH 5.5) were filter-sterilised and stored at 4°C. The hypochlorite solutions were made from a stock-solution (107,000 ppm) by dilution with sterile demi. H_2O_2 solutions were made from a 30% stock solution by dilution with sterile demineralised water. Casitone was obtained from Difco. Chloroperoxidase from Curvularia inaequalis was isolated and purified according to Van Schijndel et al., 1993.

Methodology:
Using sterile techniques, a suspension of approximately 10^8 bacteria per ml in citrate buffer pH 5.5 was made. From this suspension aliquots of 1.3 ml were added to a sterile reaction vessel, which was continuously stirred throughout the experiment with a magnetic stirrer. Then 0.5 ml of a

0.5 mg per ml casitone (in citrate buffer pH 5.5) solution, respectively 0.5 ml citrate buffer pH 5.5 were added. Subsequently 0.2 ml of V-CPO solutions of various concentrations (for calculations see Example 2) were added to the reaction vessels, in such a way that end concentrations of 3.2 ppm hypochlorite or 6.5 ppm hypochlorite were achieved. Also blank determinations were included were 0.2 ml sterile buffer was added instead 0.2 ml of a V-CPO solution. The reaction vessels were incubated at 30°C. Then 0.5 ml of a H_2O_2 solution was added (the concentration was chosen so that a fivefold molar excess of hydrogen peroxide was obtained at the end of the flux), to start the V-CPO reaction. The samples were incubated for exactly 5 min at 30°C. After this incubation period 1 ml of the reaction mixture was taken and added to 9 ml cold BSA (1% w/v) solution and put on ice immediately. This was done to stop the reaction of hypochlorite with the microorganisms. Survival as Colony Forming Units (CFU) per ml was determined by diluting the sample from 10^{-1} to 10^{-6} and plating 100 μl samples of the various dilutions on labelled BHI-agar plates. The plates were then incubated for 15-18 hours at 30°C. When there were no CFU detectable after this incubation period, the plates are incubated for another 24 hours at 30°C.

The killing efficiencies obtained was compared to the killing efficiencies obtained when the same hypochlorite amounts were added, using a micropump. This was done as follows:

Using sterile techniques, a suspension of approximately 10^8 bacteria per ml in citrate buffer pH 5.5 was made. From this suspension aliquots of 1.3 ml were added to a sterile reaction vessel, which was continuously stirred throughout the experiment with a magnetic stirrer. Then 0.5 ml of a 0.5 mg per ml casitone solution (in citrate buffer pH 5.5), respectively 0.5 ml citrate buffer pH 5.5 were added. Subsequently 0.2 ml of sterile citrate buffer pH 5.5 was

added. The reaction vessels were incubated at 30°C. Then a flux of 0.5 ml of hypochlorite solutions, yielding an endconcentration of 3.2 ppm hypochlorite respectively 6.5 ppm hypochlorite (for calculations on the concentration see Example 2) were applied over 5 min (flow: 0.1 ml per min) at 30°C. After the incubation period of 5 min 1 ml of the reaction mixture was taken and added to 9 ml cold BSA (1% w/v) solution and put on ice immediately. This was done to stop the reaction of hypochlorite with the microorganisms. Survival as Colony Forming Units (CFU) per ml was determined by diluting the sample from 10^{-1} to 10^{-6} and plating 100 μl samples of the various dilutions on labelled BHI-agar plates. The plates were then incubated for 15-18 hours at 30°C. When there were no CFU detectable after this incubation period, the plates are incubated for another 24 hours at 30°C. The results are shown in Table III.

Table III. The influence of casitone on the killing efficiencies of HClO, generated by V-CPO.

casitone concentration (mg/ml)	log reduction with 3.2 ppm hypochlorite generated by V-CPO	log reduction with 3.2 ppm hypochlorite	log reduction with 6.5 ppm hypochlorite generated by V-CPO	log reduction with 6.5 ppm hypochlorite
0.0	8.0 (= total kill)	8.2 (= total kill)	8.0 (= total kill)	8.2 (= total kill)
0.1	5.0	0.5	8.0 (= total kill)	1.0

EXAMPLE 4

Comparison between the killing efficiencies of Vanadium containing haloperoxidase and a heme-containing haloperoxidase.

Materials:

Bacterial strains used in this example were Escherichia coli NCTC 900, Streptococcus faecalis NCTC 1092 and Listeria innocua ATCC 33090 serotype 6B. Bacteria were grown for 15 to 18 hours at 30°C in Brain Heart Infusion (BHI) broth-medium. After cultivation the strains were washed two times in citrate buffer pH 5.5 (20 mM Na_3citrate-NaOH buffer + 10 mM NaCl). The bacterium suspensions were centrifuged in an Eppendorf centrifuge (14,000 rpm for 5 min), then the supernatants were removed and subsequently the bacterial pellet was resuspended in citrate buffer. This washing procedure was then repeated once more for each bacterial suspension. The twice washed bacterial suspension was then diluted with citrate buffer pH 5.5 to obtain a suspension of approximately 10^7 bacteria per ml. During the washing steps the cells were kept on ice. Buffers and BSA-solution (1% w/v in citrate buffer pH 5.5) were filter-sterilised and stored at 4°C. Chloroperoxidase from Curvularia inaequalis was purified according to van Schijndel et al., (1993). The heme-containing chloroperoxidase was obtained from Sigma (ex Caldariomyces fumago). Chloroperoxidase activity in the conversion of Cl^- to HOCl was determined spectrophotometrically in 20 mM sodium citrate buffer pH 5.5, 10 mM NaCl, 100 μM H_2O_2, 50 μM monochlorodimedone at 30°C, by following the conversion of monochlorodimedone (e 290 nm = 20.2 $mM^{-1}.cm^{-1}$) to dichlorodimedone (e 290 nm = 0.2 $mM^{-1}.cm^{-1}$). 1 Unit of chloroperoxidase is defined as the amount of enzyme that converts 1 μmol of monochlorodimedone (Sigma) per minute. The same assay (and activity definition) was used for both enzymes, in order to be able to dose the two enzymes at equeal activity.

Methodology:
Using sterile techniques a suspension of approximately 10^7 bacteria per ml in 20 mM sodium citrate buffer pH 5.5, 10 mM NaCl was made. From this suspension aliquots of 1.9 ml were added to sterile tubes. Subsequently 0.1 ml of stock solutions of vanadium-containing chloroperoxidase respectively of heme-containing chloroperoxidase were added to the tubes, in such a way that a dilution range for both enzymes was obtained. Also blank determinations were included where only sterile buffer was added instead of an enzyme stock solution. Then 0.5 ml of an 25 mM H_2O_2 stock solution was added. The sample were incubated for exactly 5 min at 30°C. After this incubation period 1 ml of the reaction mixture was taken and added to 9 ml cold BSA (1% w/v) solution and put on ice immediately. Survival as Colony Forming Units (CFU) per ml was determined by diluting the sample from 10^{-1} to 10^{-5} and plating 100 μl samples of the various dilutions on labelled BHI-agar plates. The plates were then incubated for 15-18 hours at 30°C. When there were no CFU detectable after this incubation period, the plates are incubated for another 24 hours at 30°C.

The results are presented in Table IV. The data clearly show that vanadium-containing chloroperoxidase provides a by far more effective hygienic system than the heme-containing chloroperoxidase, even when they are dosed at equal activity.

Table IV.

10 mM NaCl	vanadium chloro-peroxidase (U per ml)	heme chloro-peroxidase (U per ml)	log reduction
E coli	0.000		0.0
	0.00156		4.0
	0.0030		5.0
	0.0060		7.0(=total kill)
	0.012		7.0(=total kill)
	0.025		7.0(=total kill)
	0.050		7.0(=total kill)
	0.10		7.0(=total kill)
		0.000	0.0
		0.00156	0.1
		0.0030	0.1
		0.0060	0.1
		0.012	0.2
		0.025	1.0
		0.050	1.2
		0.10	1.2

L. innocua	0.000		0.0
	0.00156		7.0 (total kill)
	0.0030		7.0 (total kill)
	0.0060		7.0 (total kill)
	0.012		7.0 (total kill)
	0.025		7.0(total kill)
	0.050		7.0(total kill)
	0.10		7.0(total kill)
		0.00	0.0
		0.00156	0.0
		0.0030	0.0
		0.0060	0.0
		0.012	0.0
		0.025	0.0
		0.050	0.0
		0.10	0.0
S.faecalis	0.00		0.0
	0.00156		5.0
	0.0030		5.0
	0.0060		7.0(total kill)
	0.012		7.0(total kill)
	0.025		7.0 (total kill)
	0.050		7.0(total kill)
	0.10		7.0(total kill)
		0.00	0.0
		0.00156	0.0
		0.0030	0.1
		0.0060	0.1
		0.012	0.1
		0.025	0.1
		0.050	0.1
		0.10	0.9

EXAMPLE 5

The effect of various hydrogen peroxide sources on the inhibitory action of V-CPO.

In the previous examples hydrogen peroxide, which is one of the substrates in the V-CPO reaction, was added from a stock solution. In this example the effects of using other hydrogenperoxide sources is described. Oxidases were assayed at 30°C in a Biological Oxygen Monitor YSI Model 5300 (Oxygen chamber model 5301) in which oxygen consumption was monitored, using citrate buffer pH 5.5 (20 mM Na_3citrate-NaOH buffer + 10 mM NaCl) as a buffer. The Buffer were saturated with air at 30°C. As a substrate for glucose oxidases 15 $g.l^{-1}$ (end concentration) glucose was used. The bacterial strain used in this example was Escherichia coli NCTC 900. Bacteria were grown for 15 to 18 hours at 30°C in Brain Heart Infusion (BHI) broth-medium. After cultivation the strains were washed two times in citrate buffer pH 5.5 (20 mM Na_3citrate-NaOH buffer + 10 mM NaCl). The bacterium suspensions were centrifuged in an Eppendorf centrifuge (14,000 rpm for 5 min), then the supernatant were removed and subsequently, the bacterial pellet was resuspended in citrate buffer. This washing procedure was then repeated once more for each bacterial suspension. The twice washed bacterial suspension was then diluted with citrate buffer pH 5.5 to obtain a suspension of approximately 10^8 bacteria per ml. During the washing steps the cells were kept on ice. Buffers and BSA-solution (1% w/v in citrate buffer pH 5.5) were filter-sterilised and stored at 4°C. The hypochlorite solutions were made from a stock-solution (107,000 ppm) by dilution with sterile demi. H_2O_2 solutions were made from a 30% stock solution by dilution with sterile demineralised water. Casitone was obtained from Difco. Chloroperoxidase from Curvularia inaequalis was isolated and purified according to Van Schijndel et al.,(1993). Glucose oxidase ex Aspergillus niger was obtained from Sigma.

Methodology:
Using sterile techniques, a suspension of approximately 10^8 bacteria per ml in citrate buffer pH 5.5 was made. From this suspension aliquots of 1.3 ml were added to a sterile reaction vessel, which was continuously stirred throughout the experiment with a magnetic stirrer. Then 0.5 ml of a 0.5 mg per ml casitone solution (in citrate buffer pH 5.5) was added. Subsequently, 0.2 ml of V-CPO solutions were added to the reaction vessels to give a final concentration of 6.5 ppm hypochlorite. Also blank determinations were included were 0.2 ml sterile buffer was added instead 0.2 ml of a V-CPO solution. The reaction vessels was incubated at 30°C. Then 0.5 ml of one following three hydrogen peroxide generating systems were added:

1. H_2O_2 from a 3 mM stock solution;
2. sodium percarbonate from a 3 mM stock solution;
3. a mixture of glucose oxidase (0.39 Units/ml) and 75 mg/ml glucose.

The samples were incubated for exactly 5 min at 30°C. After this incubation period 1 ml of the reaction mixture was taken and added to 9 ml cold BSA (1% w/v) solution and put on ice immediately. This was done to stop the reaction of hypochlorite with the microorganisms. Survival as Colony Forming Units (CFU) per ml was determined by diluting the sample from 10^{-1} to 10^{-6} and plating 100 μl samples of the various dilutions on labelled BHI-agar plates. The plates were then incubated for 15-18 hours at 30°C. When there were no CFU detectable after this incubation period, the plates are incubated for another 24 hours at 30°C. The values obtained were compared to the MIC value that was obtained when exactly the same amounts of hypochlorite were added, using a micropump. This was done as follows:

Using sterile techniques, a suspension of approximately 10^8 bacteria per ml in citrate buffer pH 5.5 was made. From this suspension aliquots of 1.3 ml were added to a sterile reaction vessel, which was continuously stirred throughout

the experiment with a magnetic stirrer. Then 0.5 ml of a casitone solution (0.5 mg/ml in citrate buffer pH 5.5) was added. Subsequently 0.2 ml of sterile citrate buffer pH 5.5 was. The reaction vessels was incubated at 30°C. Then a flux of 0.5 ml of a hypochlorite solutions of 32.5 ppm was applied over 5 min (flow: 0.1 ml per min) at 30°C. After the incubation period of 5 min 1 ml of the reaction mixture was taken and added to 9 ml cold BSA (1% w/v) solution and put on ice immediately. This was done to stop the reaction of hypochlorite with the microorganisms. Survival as Colony Forming Units (CFU) per ml was determined by diluting the sample from 10^{-1} to 10^{-6} and plating 100 μl samples of the various dilutions on labelled BHI-agar plates. The plates were then incubated for 15-18 hours at 30°C. When there were no CFU detectable after this incubation period, the plates are incubated for another 24 hours at 30°C. The results are shown in Table V.

Table V. V-CPO reaction with different H_2O_2 sources.

hydrogen-peroxide source	casitone concentration (mg/ml)	log reduction at 6.5 ppm hypochlorite	log reduction at 6.5 ppm hypochlorite generated enzymatically by V-CPO
H_2O_2 stock solution	0.0	8.2 (= total kill)	8.0 (= total kill)
per-carbonate	0.0	8.0 (= total kill)	8.2 (=total kill)
glucose oxidase	0.0	6.4 (= total kill)	6.4 (= total kill)
H_2O_2 stock solution	0.1	1.0	8.0 (= total kill)
per-carbonate	0.1	0.8	8.2 (= total kill)
glucose oxidase	0.1	1.0	6.4 (= total kill)

<u>EXAMPLE 6</u>

Method to determine the coding sequence of the chloro peroxidase gene (cDNA) and the gene from <u>Curvularia inaequalis</u> (Centraal Bureau voor Schimmelcultures, the Netherlands, strain No 102.42) and possible expression systems.

Chloroperoxidase was isolated and purified from liquid cultures of <u>C. inaequalis</u> as described by Van Schijndel et

al., 1993), except that after DEAE chromatography two additional purification steps were performed using an FPLC system (Pharmacia LKB). First a phenyl-sepharose Cl-4B hydrophobic interaction column was used to bind the enzyme in the presence of 2 M NaCl in 50 mM Tris-HCl (pH 8.3), followed by elution with a descending gradient from 2 M NaCl in 50 mM Tris-HCl (pH 8.3). For the final purification a MonoQ HR 5/5 anion exchange column (ex Pharmacia LKB) was used to bind the enzyme, followed by elution with a gradient from 0 M to 0.5 M NaCl in 20 mM piperazine-HCl (pH 5.4). Subsequent concentration of the enzyme was carried out using rotation evaporation, followed by dialysis against 50 mM Tris-SO_4 buffer (pH 8). The purified chloroperoxidase was enzymatically digested with the proteases Staphylococcus V8 and trypsin respectively, according to standard procedures known in the art, or chemically cleaved with CNBr (Gross, E. (1967), Methods Enzymology 11, 238-255). The resulting peptides were separated using SDS-PAGE according to Laemmli (Laemmli, U.K. (1970) Nature 227, 680-685) or on a tricine gel as according to Schagger and Von Jagow (1987) and subsequently transferred to PVDF membranes (Immobilon-P ex Millipore) using CAPS transfer buffer (10 mM 3-[cyclohexylamino]-1-propanesulfonic acid, 10% methanol, pH 11) as described by Matsudaira (1987). After electrophoretic elution the membrane was rinsed for 5 min with deionized water, stained with 0.1% Coomassie Blue R-250 in 50% methanol for 5 min, and destained in 50% methanol, 10% acetic acid for 10 min at room temperature and air dried. Peptide bands were submitted to automatic Edmann sequencing on a Porton LF 3000 protein sequencer (Beckman Instruments, Inc., USA). The results of the amino acid sequence determination are summarized in Figure 1.

Based on the amino acid sequences of the peptides fully degenerated oligonucleotides were designed (see Table VII). These degenerated primers were used in Polymerase Chain

Reaction (PCR) reactions, using first-strand cDNA as a template. First-strand cDNA was prepared as follows: For the isolation of RNA spores of C. inaequalis were inoculated into a fermentation medium containing 4 g yeast extract and 2 ml of a microelement solution (Van Schijndel et al., 1993) per litre. After several days of growth the mycelium were harvested by filtration and lyophilized. The lyophilized C. inaequalis mycelia were ground under liquid nitrogen. RNA was extracted by adding an RNA extraction buffer (42 mM sodium citrate pH 7, 0.83% N-laurylsarcosine, 50 mM beta-mercaptoethanol, 1% Triton X-100 and 4 M guanidine isothiocyanate), and incubating for 1 hour at room temperature. 0.1 Volumes of 2 M sodium acetate (pH 4) and 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added and the mixture placed on ice for 15 min. After centrifugation for 10 min at 10000xg (4°C), the aqueous phase was collected, 1 volume of absolute alcohol was added and the mixture was incubated for 1 hour at -20°C followed by brief centrifugation at 10,000xg. The pellet was resuspended in an appropriate volume of RNA extraction buffer and fractionated by ultracetrifugation in a gradient of cesium chloride (Sambrook et al., 1989). The pellet was carefully washed and stored in a 75% ethanol solution at -70°C. For mRNA isolation the RNA was precipitated and resuspended in RNAse free water after which the mRNA was extracted using the polyAtract mRNA isolation kit (Promega corporation, USA). First strand cDNA synthesis was carried out on isolated mRNA from C. inaequalis using the Pharmacia first-strand cDNA synthesis kit (Pharmacia Biotech). Four 20-meric degenerated oligonucleotides were designed based on the amino acid sequences of chloroperoxidase peptides (see also Table VII) and used as primers in polymerase chain reactions with first strand cDNA from C. ineaqualis as a template. Polymerase chain reactions were performed using a thermocycler (Eppendorf mastercycler 5330) and Taq polymerase (Promega corporation). For optimal amplification of the chloroperoxidase encoding cDNA using the degenerated primers the polymerase chain reaction was performed at 46°C

(annealing step) for 30 cycles. The two resulting specific fragments were ligated into a pUC18 vector, cloned and sequenced from both strands. Based on the DNA sequence results the following two specific primers were designed:

5'- CATAGCGATAGCGACGCGGA-3'

and

5'- CTAACCCCGGCGCCAACATC-3'

These two primers were used in polymerase chain reactions with first strand cDNA as a template. Thus a gene-specific DNA fragment was obtained joining the two known DNA sequences. This fragment was cloned in a pUC18 vector and subsequently sequenced. To obtain the 5' region of the mRNA encoding the chloroperoxidase the 5'-Amplifinder RACE kit (Clonetech corporation) was used. The genomic chloroperoxidase gene from <u>C. ineaqualis</u> was isolated as follows:

<u>C. ineaqualis</u> genomic DNA was isolated from lyophilized mycelia which was ground under liquid nitrogen and extracted with an appropriate amount of extraction buffer (200 mM Tris-HCl, pH 8.5, 25 mM EDTA, 250 mM NaCl, 1% SDS and 0.2 mg per ml proteinase K). After incubation overnight at room temperature 0.7 volumes of phenol and 0.3 volumes of chloroform were added and mixed vigorously. The tubes were centrifuged at 10,000xg and the aqueous layer was transferred to a clean tube. The genomic DNA was precipitated with 2 volumes of absolute ethanol. After centrifugation for 5 min at 5000xg the pellet was resuspended in 2 ml of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA and treated with RNAse (Boehringer Mannheim) as recommended by the manufacturer. The genomic DNA containing solution was extracted with phenol:chloroform:isoamylalcohol (25:24:1) and after ethanol precipitation, finally dissolved in a suitable volume of 10 mM Tris-HCl pH 8, 1 mM EDTA buffer. For Southern analysis of the genomic DNA, the

DNA was digested with several combinations of restriction enzymes and after agarose gel electrophoresis blotted to a nitrocellulose membrane (Sambrook et al., 1989). Hybridization was carried out using a radiolabeled gene specific fragment (amplified with the two specific primers described above), which was made by random priming using alpha-32P labelled dATP (Sambrook et al., 1989). Based on the results obtained a mini-library was made using genomic DNA digested with Pst I which was inserted in the vector pUC18. The library was screened with the same probe as described for the Southern blot. A positive clone was isolated and also partly sequenced from both strands to confirm the cDNA sequence results. The C. inaequalis chloroperoxidase encoding gene and its putative gene product are disclosed in Figure 2.

A chloroperoxidase production system in Saccharomyces cerevisiae was made as follows:

The well known GAL1 inducible yeast promoter was obtained as an EcoR1 BamH1 fragment from the S. cerevisiae wild type GAL1 gene (Molecular and Cellular Biology 10, 4757-4769, 1990) and cloned into the EcoR1 BamH1 sites of respectively plasmid YCplac33 and YEplac95 (Gietz and Sugino (1988) Gene 74, 527-534). The plasmids obtained were named TNT1 respectively TNT2. A BamH1 restriction site was created in front of the 5' begin of the C. inaequalis chloroperoxidase gene by performing a PCR experiment using as a template the PstI EcoRI 5' fragment of the C. inaequalis chloroperoxidase gene subcloned into pUC18 and as primers the M13/pUC 22-mer reverse sequence primer and the primer :

5' GAG AGA GGA TCC ACT CAC TAC TTA CAA TCA CAC 3'

The amplified fragment was digested with BamHI and EcoRI. The EcoRI PvuII fragment from the C. inaequalis chloroperoxidase gene, containing the 3' part of the gene, was subcloned into EcoRI SmaI digested pUC18. From this clone, after digesting it with EcoRI and XbaI, a fragment

containing the 3' part of the C. inaequalis chloroperoxidase gene was purified.
A three point ligation was performed, which comprised either TNT1 or TNT2 digested each with BamHI and XbaI, and the 5' BamHI EcoRI fragment and the 3' EcoRI XbaI fragment. After ligation and cloning the obtained plasmids were checked for their identity. Plasmids thus obtained were named TNT3 (derived from TNT1) respectively TNT4 (derived from TNT2).
Yeast strain BJ1991 was transformed with plasmids TNT3 respectively TNT4 according to procedures known in the art and selected for ura+ transformants. Ura+ transformants were replicated to YP-plates containing either glucose (2%) or galactose (2%). After growth some cells were taken from the plates and resuspended in 200 μl 20 mM Tris-HCl pH 8.1. After incubation for 5 minutes 10 μl was taken from the fluid and spotted on a nitrocellulose filter. The nitrocellulose filters were incubated in 100 mM sodium acetate buffer (pH 5.5), 1 mM orthodianisidine, 100 mM KBr and 2 mM H_2O_2. A clear colour formation was observed on all spots derived from galactose grown yeast strains, whereas no colour formation was observed for the glucose grown yeasts. This indicates that a galactose inducible production system for the Curvularia inaequalis chloroperoxidase gene in the yeast Saccharomyces cerevisiae has been constructed. A similar assay, making use of 100 mM potassium phosphate buffer (pH 6.5), 100 mM KBr, 1 mM H_2O_2 and 40 μM phenol red (BDH), clearly showed a blue/purple colour formation with fluids from galactose grown yeast, whereas no colour changes occurred with fluids from glucose grown yeasts. To further confirm that a heterologous chloroperoxidase gene expression system in yeast was made that produced C. inaequalis chloroperxidase with the same functionality as the C. inaequalis chloroperoxidase, the recombinant enzyme was purified from galactose induced TNT3 or TNT4 transformed yeast strains. After growth in galactose containing medium, yeast cells were harvested and

resuspended in 20 mM Tris-HCl (pH 8.1). Sterile glass beads were then added and the suspension was shaken vigorously. After centrifugation for 15 minutes at 10,000xg the supernatant was taken and applied to a DEAE column as the recombinant enzyme was purified using essentially the purification protocol as for the wild type C. inaequalis enzyme (see above). After purification a recombinant chloroperoxidase was obtained with a specific activity of 22 U per mg protein (determined in 100 mM sodium acetate buffer pH 5.0, 1 mM H_2O_2, 5 mM potassium chloride and 50 μM MCD, see also van Schijndel et al., 1993), which compares very well to the specific activity of approximately 20 U per of protein that was obtained with purified (see above) chloroperoxidase from C. inaequalis itself. The pH activity profiles of the wild type chloroperoxidase and the recombinant chloroperoxidase derived from yeast are shown in Figure 3. Figure 3 provides further evidence that the recombinant yeast produced enzyme has the same functionality as the wild type enzyme.

EXAMPLE 7
Screening for suitable haloperoxidases in other microorganisms.

The microorganisms used in this example are Curvularia inaequalis (CBS 102.42), Drechslera biseptata (CBS 371.72), Drechslera fugax (CBS 509.77), Drechslera nicotiae (CBS 655.74), Drechslera subpapendorfii (656.74), Embelisia hyacinthi (416.71), Embelisia didymospora (CBS 766, Ulocladium chartarum (200.67) and Ulocladium botrytis (452.72). Various fungi are grown on agar plates. When the growth is completed the extracellular proteins are transferred (replica blotted) to a nitrocellulose filter, which was prewetted in 50 mM Tris-HCl buffer (pH 8.3). After 15 minutes of incubation on the agar plates, the filter was tested for haloperoxidase activity by soaking the filter in 100 mM sodium acetate (pH 5.5) or potassium

phosphate (pH 6.5 and 7.5), 1 mM orthodianisidine, 2 mM hydrogen peroxide in the presence and absence of 0.1 M potassium bromide. Thus the production of a bromoperoxidase and/or chloroperoxidase can be detected. To test whether the produced haloperoxidase is a vanadium-containing haloperoxidase, the test described above was repeated in the presence and absence of 10 and 100 μM sodium vanadate. In the case of vanadium-containing haloperoxidases an increase in signal could be observed in situations where vanadate was supplemented. To test whether the chloroperoxidases identified are indeed similar to the vanadium haloperoxidase from <u>C. inaequalis</u>, small amounts of chloroperoxidases were purified (essentially as described in van Schijndel <u>et al</u>., 1993) from <u>Ulocladium chartarum</u>, <u>Embelisia didymospora</u> and <u>Drechslera subpapendorfii</u>. The pH optima of these enzymes varied from pH 4.5 -5.5. The chlorinating activity of these enzymes increased upon addition of vanadate, which clearly indicates that these enzymes are indeed vanadium haloperoxidases. To further test the similarity of the enzymes identified with <u>C. inaequalis</u> vanadium-chloroperoxidase, one of the haloperoxidases identified was further characterised. For this the chloroperoxidase produced by the fungus <u>Drechslera biseptata</u> (CBS 371.72) was chosen. It has properties similar to the chloroperoxidase from <u>Curvularia inaequalis</u> that is high thermostability and high affinity for its substrates. The EPR spectrum of the purified enzyme was also recorded. As for other vanadium haloperoxidases (de Boer et al., 1988; Wever et al., 1988), the oxidized enzyme is EPR silent; however upon reduction with sodium dithionite a typical vanadyl EPR spectrum was observed (data not shown). The isotropic EPR parameters g_o of 1.969 and A_0 of 9.0 mT are almost the same as those found for the enzyme from <u>C. inaequalis</u> (Wever et al, 1985). Furthermore, the purified enzyme has been split into peptides using proteases and cyanogen bromide. The peptide maps show the same patterns suggesting that these two enzymes have a large sequence

homology. This is indeed the case, two separated peptides of the enzymes which were obtained by treatment of the enzyme with a protease and which were purified, were sequenced. The sequences show a very large homology and therefore it can be concluded that the two enzymes are very similar.

Amino acid sequence of a peptide from C. inaequalis :
(Asp)-leu-arg-gln-pro-tyr-asp-pro-thr-ala-pro-ile-glu-asp-gln-pro-gly-ile-val-arg-thr-

Amino acid sequence of a similar peptide from D. biseptata

Asp-leu-arg-gln-pro-tyr-asp-pro-thr-ala-pro-ile-glu-glu-gln-pro-gly-ile-val-arg-thr-

Suitable vanadium containing haloperoxidases can thus be identified by using a replica technique in which the increase in activity after addition of vanadate is tested, and/or by (partially) purifying the enzyme and looking for increase in activity after addition of vanadate.

EXAMPLE 8

Further screening for suitable chloroperoxidases in other microorganisms, using antibodies.

The strains used in this example are: Curvularia inaequalis (CBS 102.42), Drechslera biseptata (CBS 371.72), Drechslera subpapendorfii (CBS 656.74), Embellissia didymospora (CBS 766.79) and Ulocladium chartarum (CBS 200.67).
The microorganisms were grown in two phases. First, 50 ml of sterile germination medium (as described in Van Schijndel, et al., 1993) was inoculated with the spore mass of the microorganisms. The culture was shaken for 3 days at 23°C after which the culture was transferred to a 3 litre Erlenmeyer flask containing 1 litre of fermentation medium (5 g of casein hydrolysate (Gibco BRL), 3 g of yeast extract and 1 g of fructose per litre of deionized water).

The medium, which was shaken at 23°C, was collected after 14-17 days, filtered and the chloroperoxidases were purified essentially according to Van Schijndel et al. (1994). Polyclonal antibodies were raised (using Freunds complete adjuvans in the first injection and Freunds incomplete adjuvans in the booster injection) against the purified (according to van Schijndel et al., 1994) chloroperoxidase from Curvularia inaequalis in a (2-month-old female) rabbit. The rabbit was bled 6 days after the last booster injection. The sera were heated for 30 min at 56°C to inactivate complement and then centrifuged. The supernatant was taken. A dilution series was made of each of the purified chloroperoxidases and also of bromoperoxidase from Ascophylum nodosum (purified according to Wever et al, 1985), starting with 50 μl of each protein (of approximately 0.1 mg per ml), and each sample was diluted sequentially two times. The dilutions were spotted using a dot-blot apparatus (Bio-Rad) on a nitrocellulose filter, washed with 2% BSA and incubated sequentially with a 1:800 dilution of rabbit anti-chloroperoxidase antiserum, biotinylated goat anti-rabbit (dilution 1:3000), alkaline phosphatase-conjugated streptavidin (dilution 1:2000) and colour development reagent (5-bromo-4-chloro-3-indolyl phosphate, 4-NitroBlue Tetrazolium chloride (Boehringer Mannheim). All steps were performed according to standard protocols. The results obtained are shown in Table VI.

Table VI

haloperoxidase from	cross-reactivity with polyclonal antibodies raised against C. inaequalis chloroperoxidase
C. inaequalis	yes
D. biseptata	yes
D. subpapendorfii	yes
E. didymospora	yes
U. chartarum	yes
bromoperoxidase from A. nodosum	no

Based on the results disclosed in Table VI it can be concluded that immunoassays with antibodies raised against C. inaequalis chloroperoxidase is suitable to identify other suitable vanadium containing haloperoxidases. These haloperoxidases can be either in crude form, partially or totally purified. Purification techniques can be all techniques known in the art, like gelfiltration, ion exchange chromatography, hydrophobic interaction chromatography, precipitation techniques, (ultra)filtration techniques, affinity chromatography, gelelectrophoresis and others.

EXAMPLE 9

Further screening for suitable chloroperoxidases in other microorganisms.

In this example it si described how a radio-active probe derived from the chloroperoxidase gene from Curvularia inaequalis can be used to detect homologous genes in other microorganisms. This was done as follows:

Chromosomal DNA from C. inaequalis (CBS 102.42), Embelissia didymospora (CBS 766.79) and, Drechlera biseptata (CBS 371.72) was purified essentially as described for C. inaequalis chromosomal DNA (as described in example 6). For Southern analysis of the genomic DNA, the DNA was digested with several combinations of restriction enzymes and after agarose gel electrophoresis blotted to a nitrocellulose membrane (Sambrook et al., 1989). Hybridization was carried out using a radiolabeled gene specific fragment, which was made by random priming using alpha-^{32}P labelled dATP (Sambrook et al. 1989). The gene specific fragment used was amplified (before radio-active labelling) in a polymerase chain reaction, using first strand cDNA (see Example 6) as a template and making use of the primers:

5'-CACGATGGGGTCCGTTACAC

and 5'-GTACCGCTATCGCTGCGCCTG

The hybridisation conditions were as follows:
In the prehybridisation and hybridisation 6 * SSPE, 5 * Denhardts, 0.5% SDS and 10 mg salmon sperm DNA were used as a buffer. Prehybridisation was done for 1 hour at 55°C, then the radioactive probe was boiled for 1 min and then directly added. Subsequently, hybridisation was continued overnight. The autoradiograph, which was obtained with Curvularia inaequalis and Drechlera biseptata DNA is presented as Figure 4. In the figure, lane 1: lambda DNA; lane 2: non-digested C. inaequalis genomic DNA; lane 3: idem, digested with EcoRI; lane 4: digested with BamHI; lane 5: EcoRI and BamHI; lane 6 digested with XbaI; lane 7 PstI; lane 8 XbaI and PstI; lanes 9 - 14: idem, using D. biseptata. As can be seen in the figure, a positive signal is obtained with chromosomal DNA from Drechslera biseptata, indicating a high degree of similarity of both genes. Similar results were obtained with DNA from Embellisia didymospora. We therefor conclude that the chloroperoxidase gene, or parts derived from this gene, or probes based on

the sequence of the C. inaequalis chloroperoxidase gene can be used to detect suitable vanadium haloperoxidases from other microorganisms.

Table VII. Oligonucleotide primers (20mers), based on amino acid sequences from the vanadium chloroperoxidase ex Curvularia inaequalis.

I: inosine

A/G: at this position at equal mix of A and G is used.

C/T: at this position at equal mix of C and T is used.

G/A/T/C: at this position at equal mix of G, A, C and T is used.

Oligo 1:

5'-T A C/T A T G A A A/G C C I G T I G A A/G C A -3'

Oligo 2:

5'-A G/A T/C T G I G C G/A T A I G C G/A T T G/A T C-3'

Oligo 3:

5'-G A C/T G A A/G A C I G C I G A A/G T A C/T G A-3'

Oligo 4:

5'-A G/A I G C T/C T G I G C I C C G/A/T/C C C C A T-3'

CLAIMS

1. Enzymatic antimicrobial composition comprising a Vanadium haloperoxidase, a source of halide and hydrogen peroxide or a source of hydrogen peroxide.

2. Enzymatic antimicrobial composition according to claim 1, wherein the Vanadium haloperoxidase is a chloroperoxidase.

3. Enzymatic antimicrobial composition according to any one of the preceding claims, wherein the Vanadium haloperoxidase is a chloroperoxidase obtainable from Curvularia inaequalis

4. Enzymatic antimicrobial composition according to any one of the preceding claims, wherein the Vanadium haloperoxidase is a chloroperoxidase which is immunologically cross-reactive with the chloroperoxidase from Curvularia inaequalis CBS 102.42.

5. Enzymatic antimicrobial composition according to any one of the preceding claims, wherein the source of hydrogen peroxide is an enzymatic hydrogen peroxide-generating system.

6. Enzymatic antimicrobial composition according to claim 5, wherein the enzymatic hydrogen peroxide-generating system is glucose/glucose oxidase or lactate/lactate oxidase.

7. Enzymatic antimicrobial composition according to any one of the preceding claims, said composition being essentially free from catalase activity.

8. Process for inhibiting microbial growth, comprising applying a composition according to any one of the preceding claims to a surface which is to be disinfected.

9. Use of Vanadium haloperoxidases as sanitizing agent.

10. DNA sequence comprising a structural gene coding for Vanadium chloroperoxidase from Curvularia inaequalis CBS 102.42.

11. DNA sequence comprising a structural gene for Vanadium chloroperoxidase from Curvularia inaequalis CBS 102.42 as shown in Figure 2.

12. Expression vector comprising an origin of replication, transcription and termination control sequences and at least part of the DNA sequence according to claim 10 or 11.

13. Process for preparing a Vanadium haloperoxidase by transforming a suitable host by means of an expression vector according to claim 12, cultivating the host under conditions which allow the expression of the structural gene and isolating the Vanadium haloperoxidase.

*****

Fig.1.

Peptide sequences derived from vanadium chloroperoxidases

sequence	cleavage method
C. inaequalis	
1ML--LYMKPVEQPNPNPGANISDNAYAQLGLVLDRSVLEA[a]	CNBr
2(S)NADETAEYDDAVRVAIAMGGAQALNS[a]	Trypsin
3(G)YHPTPGRYKFDDEP	Trypsin
4IDEPEEYN	Trypsin
5**(D)LRQPYDPTAPIE**D**QPGIV**RT[b]	Trypsin
D. biseptata	
6LNGLNR**DLRQPYDPTAPIE**E**QPGIV**[b]	V8 prot.

[a]underlined sequences are used to design degenerated DNA primers.
[b]homologous sequences between *C. inaequalis* and *D. biseptata* are in bold.

Fig.2.

```
ATGGGGTCCG TTACACCCAT CCCACTCCCT AAGATCGATG AACCCGAAGA GTACAACACC   60
AACTACATAC TATTCTGGAA CCATGTCGGT TTGGAACTCA ACCGCGTAAC TCACACTGTT  120
GGAGGCCCCC TGACGGGACC ACCTCTCTCT GCCAGGGCTC TGGGTATGCT GCACTTGGCT  180
ATTCACGACG CCTACTTTTC TATCTGCCCT CCGACCGACT TCACCACCTT CCTCTCACCT  240
GATACTGAGA ATGCCGCGTA CCGTCTACCT AGCCCTAATG GTGCAAATGA TGCTCGCCAA  300
GCAGTCGCTG GAGCTGCCCT CAAGATGCTG TCTTCACTGT ACATGAAGCC CGTCGAGCAG  360
CCTAACCCTA ACCCCGGCGC CAACATCTCC GACAACGCTT ATGCTCAGCT TGGCTTGGTT  420
CTCGACCGAT CAGTTCTGGA GGCACCTGGT GGCGTGGACC GAGAGTCAGC CAGTTTCATG  480
TTTGGTGAGG ATGTAGCCGA TGTCTTCTTC GCACTCCTCA ACGATCCTCG AGGTGCTTCG  540
CAGGAGGGCT ACCACCCTAC ACCCGGCCGC TATAAATTTG ACGATGAACC TACTCACCCT  600
GTCGTCCTCA TTCCAGTAGA CCCCAACAAC CCTAATGGTC CCAAGATGCC TTTCCGTCAG  660
TACCACGCCC CATTCTACGG CAAGACCACG AAGCGTTTTG CTACGCAGAG CGAGCACTTC  720
CTGGCCGACC CACCGGGCCT GCGTTCTAAT GCGGACGAGA CCGCGGAGTA TGACGACGCC  780
GTCCGCGTCG CTATCGCCAT GGGTGGTGCT CAGGCTCTCA ACTCCACCAA GCGTAGCCCA  840
TGGCAGACAG CACAGGGCCT ATACTGGGCC TACGATGGGT CAAACCTCAT TGGCACACCA  900
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CCTCGCTTTT ACAACCAGAT CGTACGTCGC ATCGCAGTTA CGTACAAGAA GGAAGAGGAC    960
CTTGCCAACA GCGAAGTCAA CAATGCGGAT TTCGCCCGCC TCTTCGCCCT CGTCGACGTC   1020
GCTTGCACAG ACGCTGGTAT CTTTTCCTGG AAGGAGAAAT GGGAGTTCGA ATTCTGGCGC   1080
CCACTATCTG GTGTGCGAGA CGACGGCCGT CCAGACCATG GAGATCCTTT CTGGCTCACT   1140
CTCGGTGCCC CAGCTACTAA CACCAACGAC ATTCCATTCA AGCCTCCTTT CCCAGCTTAC   1200
CCATCTGGTC ACGCGACCTT TGGCGGTGCT GTGTTCCAAA TGGTGCGTCG ATACTACAAC   1260
GGCCGCGTAG GTACATGGAA GGACGACGAA CCCGACAACA TTGCCATCGA TATGATGATC   1320
TCGGAGGAGC TCAACGGCGT GAACCGCGAC CTACGCCAGC CTTATGACCC CACGGCCCCA   1380
ATCGAAGACC AACCCGGTAT CGTGCGCACC CGCATTGTTC GCCACTTCGA CTCGGCCTGG   1440
GAACTCATGT TCGAAAACGC CATTTCGCGC ATCTTCCTCG GTGTCCACTG GCGTTTCGAT   1500
GCCGCCGCCG CCCGCGACAT TCTCATTCCC ACGACGACAA AGGACGTCTA CGCTGTCGAC   1560
AACAATGGCG CCACCGTGTT CCAGAACGTA GAGGACATTA GGTACACAAC CAGGGGTACG   1620
CGTGAGGACC CCGAGGGCCT CTTCCCTATC GGTGGTGTGC CACTGGGTAT CGAGATTGCG   1680
GATGAGATTT TTAATAATGG ACTTAAGCCT ACGCCCCCGG AGATCCAGCC TATGCCGCAG   1740
GAGACACCGG TGCAGAAGCC GGTGGGACAG CAGCCGGTTA AGGGCATGTG GGAGGAAGAG   1800
CAGGCGCCGG TAGTCAAGGA GGCGCCGTAG                                    1830
```

Fig.2. (Cont.)

Fig.3.

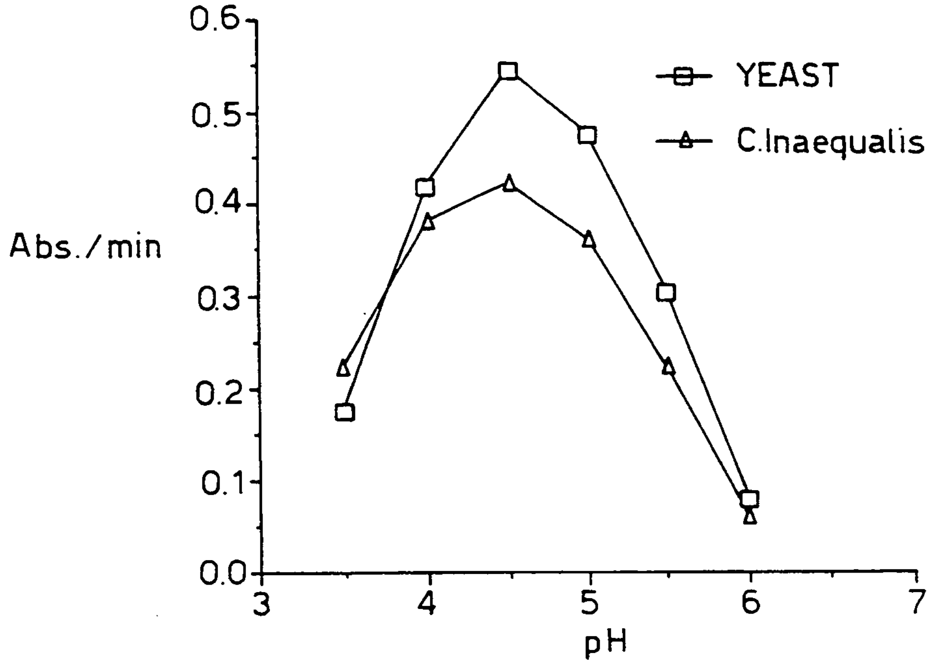
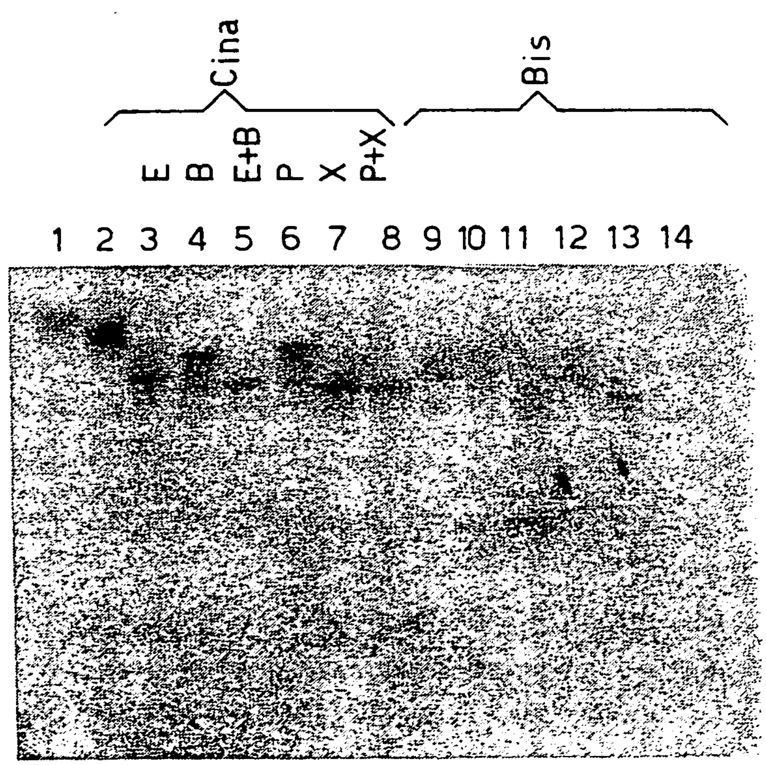

0.6
0.5
0.4
0.3
0.2
0.1
0.0
Abs./min
3
4
5
6
7
pH
YEAST
C.Inaequalis

Fig.4.


Cina
Bis
E
B
E+B
P
X
P+X
1 2 3 4 5 6 7 8 9 10 11 12 13 14